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Mean d^2 and Divergence Time: Transformations and Standardizations

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The fitness consequences of inbreeding and outbreeding have intrigued biologists for a long time. Recently a measure of relatedness of parental haplotypes has been proposed called mean d^2. This measure is based on a stepwise mutational process and therefore is tailored to microsatellite genetic markers. Theoretical work suggests that mean d^2 typically is less suited for measuring fitness consequences due to close inbreeding rather than heterozygosity. However, mean d^2 may be more appropriate than heterozygosity for measuring divergence times over longer time scales and thus for detecting outbreeding depression. Here, simulations are used to (1) identify appropriate standardization coefficients and transformations for mean d^2, and (2) evaluate mean d^2 as a measure of divergence time of parental lineages over time scales up to 10,000 generations. Results show that mean d^2 is a linear predictor of divergence time. The coefficient of variation of mean d^2 approaches a constant value with increasing divergence time and therefore logarithm transformation is appropriate to restore homoscedasticity. When mutation rates and sizes are known for each locus they can be incorporated into a standardization coefficient to increase the precision of mean d^2. As few as 10 loci can explain more than 70% of the variation in divergence time between lineages. While heterozygosity outperforms mean d^2 at detecting differences in divergence time over relative short time periods ($\leq$1000 generations), mean d^2 can outperform heterozygosity at detecting differences over longer time periods ($\geq$1000 generations). However, gene flow of as little as 1% per generation can significantly reduce the ability of either mean d^2 or heterozygosity to estimate divergence time.

The fitness of sexually reproducing (diploid) individuals is often a function of the degree of relatedness of their parents (Thornhill 1993). Close inbreeding can result in expression of recessive deleterious mutations, reducing the fitness of inbred individuals (reviewed by Keller and Waller 2002), while outbreeding depression can result from the disruption of local adaptations or coadapted gene complexes (Templeton 1986). As such, there can be an optimal degree of relatedness of parents on the inbreeding-outbreeding continuum which maximizes fitness of their offspring (Marshall and Spalton 2000; Waser and Price 1989, 1994), and thus accurate indices are needed to measure the relatedness of parental lineages at both ends of the genomic divergence continuum.

Multilocus heterozygosity typically has been used to measure close inbreeding (Thornhill 1993). More recently, an alternative index of relatedness called mean d^2 (md^2) has been proposed (Coltman et al. 1998; Coulson et al. 1998). Mean d^2 is based on the stepwise mutation model, which may characterize many microsatellite loci, whereby alleles either increase or decrease by a single repeat (Valdes et al. 1993; Weber and Weber 1993; see also Huang et al. 2002; Xu et al. 2000). The basic premise underlying md^2 is that over evolutionary time, two independent alleles will tend to "wander" away from each other with respect to their repeat number—that is, one allele might tend to increase while the other decreases. The rate at which the alleles wander away follows the square root function. Thus a linear measure of divergence time of two alleles can be calculated from:

$$md_i^2 = \frac{1}{L} \cdot \sum (p_{il} - q_{il})^2, \qquad (1)$$

where L is the number of loci used to genotype individual i, p_{il} and q_{il} are the lengths in repeats of the two alleles of individual i at locus l, and the summation is over all loci.

Mean d^2 was derived from the established parameter D (Goldstein et al. 1995) and its analogue S_w (Slatkin 1995), both components of R_{ST}, which measures the time of divergence between two populations. The parameters D and S_w calculate the average squared difference in allele lengths at a locus between two populations, whereas md^2 calculates the squared difference in the allele lengths at a locus between two homologues within an individual. Goldstein et al. (1995) showed that D provides a positive linear measure of the divergence time of two populations. Furthermore, Slatkin

(1995) shows that linearity is maintained even when considering multistep mutations, which occur at some microsatellite loci (Di Rienzo et al. 1994; Huang et al. 2002). However, when a constraint on allele size (e.g., Deka et al. 1999; Garza et al. 1995; Huang et al. 2002; but see Falush and Iwasa 1999) is modeled, linearity is eventually lost as the populations become saturated with all possible allele sizes. Goldstein et al. (1995) showed that this occurs at a predictable time, which depends on the mutation rate and the maximum allowable size of the microsatellite locus (see their Equation 4). Presumably these results equally pertain to md^2.

An accumulating number of studies have found a positive correlation between md^2 and various components of fitness (reviewed by Coltman and Slate 2003). For example, in harbor seals (*Phoca vitulina*), birth weight and neonatal survivorship were positively correlated with md^2 (Coltman et al. 1998), and other positive relationships have been reported in horseshoe bats (*Rhinolophus ferrumequinum*; Rossiter et al. 2001), great reed warblers (*Acrocephalus arundinaceus*; Hansson et al. 2001), black grouse (*Tetrao tetrix*; Höglund et al. 2002), Lipizzan horses (Curik et al. 2003) and two salmonid species (*Salmo salar* and *Salvelinus alpinus*; Primmer et al. 2003). Two studies have alternatively found a negative relationship (Garnier-Gere et al. 2002; Marshall and Spalton 2000), indicating outbreeding depression. Coulson et al. (1998) showed that md^2 was positively correlated with birth weight and neonatal survivorship in red deer calves (*Cervus elaphus*). However, based on a larger set of microsatellite loci, Slate and Pemberton (2002) later were unable to confirm the relationship. While the populations of red deer studied were similar, the population studied by Slate and Pemberton (2002) was largely descended from a single stag. Yet other studies have found no relationship (e.g., Amos et al. 2001; Rowe and Beebee 2001; Shikano and Taniguchi 2002; Weatherhead et al. 2002), and theoretical work done by Tsitrone et al. (2001) has called into question whether md^2 is more informative then heterozygosity, at least at detecting inbreeding depression (see also Coltman and Slate 2003; Goudet and Keller 2002).

Tsitrone et al. (2001) examined close inbreeding by modeling a single population where some individuals self-fertilized, while the remaining individuals mated randomly. Fitness (and inbreeding depression) was then calculated as a decreasing linear function of the number of selfing events in an individual's pedigree. They found that heterozygosity outperformed md^2 as an index of fitness. For example, by examining a single locus with a mutation rate of 10^{-2}, a population size of 1000, and a selfing rate of 0.4, they found that the correlation coefficient between heterozygosity and fitness was about 0.60, while the coefficient for md^2 was only 0.16.

Tsitrone et al. also examined deep inbreeding by splitting a large population into two smaller populations and allowing these subpopulations to breed for a number of generations. The subpopulations were then brought back together and allowed to interbreed. Fitness was calculated as a linear function of the proportion of alleles that had equivalent subpopulation origin (assuming full recombination). For example, after admixture, the least fit class of individuals would have pure parental origin, having never "outcrossed." In this case, md^2 was a better predictor of fitness than heterozygosity only when the product of mutation rate and subpopulation size was greater than one. A recent meta-analysis also shows that heterozygosity typically outperforms md^2 at measuring inbreeding depression, although there was considerable heterogeneity in the effectiveness of md^2 among studies and numerous correlations were negative, indicating outbreeding depression and not inbreeding depression (Coltman and Slate 2003).

This article revisits the original formulation of md^2 as a measure of divergence time of parental lineages. Computer simulations are developed that incorporate population size; mutation rate, including length-dependent mutations; and mutational mechanism (stepwise mutation model [SMM] and two-phase mutation model [TPM]). The simulations are used to determine the transformations to restore homoscedasticity, appropriate standardization coefficients, and the number of loci required to precisely calculate divergence time. Comparisons are made between the abilities of md^2 and heterozygosity to predict divergence times over a range up to 10,000 generations. Finally, the effect of gene flow on the abilities of both md^2 and heterozygosity to estimate divergence time is examined.

Methods

Transformations and Standardization for md^2

Simulations were run based on a set of parameters comprising the population size ($N = 10^2$, 10^3, or 10^4), the number of loci (10 or 50), the mutation rate ($\mu = 10^{-3}$ or 10^{-4}), and the mutational mechanism (SMM or TPM). These rates were selected to represent those commonly reported for microsatellite loci (Jarne and Lagoda 1996). Each simulation began with 100 independent populations. Allele sizes were assigned an arbitrary length and the populations were allowed to "evolve." Within each population, offspring were produced by randomly mating the parents and replicating one of each of their two alleles (selected with equal probability) with a potential for mutation that either increased or decreased the allele's length with probability $\mu/2$. For the SMM, a mutation increased or decreased the number of repeats by one, while for the TPM, 20% of all mutations were of multiple repeats. Multistep mutations occurred with frequencies defined by the geometric distribution $2^{-j} + 0.0001$, where the associated change in repeat number was $j + 1$ (Di Rienzo et al. 1994). Thus multistep mutations were of at least 2 repeats units and never more than 11 repeats.

After $10N$ generations, at which point there was a mutation-drift equilibrium and the average md^2 within each population was stable, the populations were each replicated to produce 100 pairs of populations. The pairs of populations were allowed to evolve for an additional 10,000 generations with no gene flow between them. At given generations, the average md^2 of "inbred" offspring was calculated for each population pair by randomly mating (with

replacement) two parents selected from the same population, repeated for a total of N offspring. The average md^2 of "outbred" (hybrid) offspring was calculated for each population pair by randomly mating two parents, one selected from each population, repeated for a total of N offspring.

For a subset of simulations, the mutation rate was varied across the loci such that half had a mutation rate of 10^{-3} and the other half had a mutation rate of 10^{-4}. A length-dependent mutation rate was also considered whereby longer alleles were more likely to mutate than smaller alleles. In this case, the per repeat mutation rate was set at 10^{-5} (Webster et al. 2002). Thus, as an example, an allele of 1 repeat would have a mutation rate of 10^{-5}, while an allele of 100 repeats would have a mutation rate of 10^{-3}. The populations were initialized with 10 loci having alleles ranging from 5–50 in increments of five repeats. The alleles were not allowed to drop below one repeat (i.e., alleles of one repeat could only increase to two repeats). For simulations where mutation rates varied within or across loci, the following standardized md^2 was also calculated:

$$smd_i^2 = \frac{1}{L}\sum \frac{(p_{il} - q_{il})^2}{\alpha_l}, \qquad (2)$$

where α_l is the mutation rate at locus l, or in the case of the length-dependent mutation simulation, α_l was calculated for each individual from the mean allele size ($=\frac{1}{2}[p_{il} + q_{il}]$).

The variation in md^2 as a function of divergence time was characterized using the coefficient of variation (standard deviation/average md^2 across the 100 pairs of populations), from which appropriate transformations were determined. Next, the precision and accuracy of md^2 and smd^2 were compared to determine the importance of the standardization coefficients.

Mean d^2, Heterozygosity, and Divergence Time

To examine the effectiveness of md^2 and heterozygosity at calculating divergence time, three comparisons of these two measures were made using Pearson correlation: (1) between inbred offspring (those whose parents came from the same population) and hybrid offspring (those whose parents came from different populations) after 1000 generations post-divergence of the population pairs; (2) between inbred offspring and hybrid offspring after 10,000 generations postdivergence; and (3) between hybrid offspring after 1000 generations postdivergence and hybrid offspring after 10,000 generations postdivergence. The first set of offspring in each comparison was coded as a 1 and the second set was coded as a 2. Prior to the correlation analysis, to restore normality in the data, md^2 was log + 1 transformed and heterozygosity was arcsine square root transformed.

Finally, to examine the effect of gene flow on the strengths of these correlations, the simulations were repeated with a migration rate of 1% per generation between each population pair.

Results

Transformations and Standardization for md^2

The average md^2 of hybrid offspring was linearly related to the time of divergence, regardless of the population size, mutation rate, or mutational mechanism. The slope of the line was directly proportional to the mutation rate (Figure 1). The variation around the line was primarily dependent on the number of loci used; more loci explained a greater amount of variation. For example, when 10 loci were used, each with a mutation rate of 10^{-3}, the linear relationship explained 73% of the variation for $N = 100$ (Figure 1a), 65% for $N = 1000$, and 20% for $N = 10{,}000$. When 50 loci were used, the linear relationship explained 93%, 91%, and 35% for the three respective population sizes.

The variation around the linear relationship between md^2 and divergence time was also affected by the mutation rate, but not by the mutational mechanism. When 10 loci, each with mutation rates of 10^{-4} were used, the explained variation was lower, at 68% for $N = 100$ (Figure 1b), 62% for $N = 1000$, and 11% for $N = 10{,}000$. When 10 loci following the TPM with mutation rates of 10^{-3} were examined, the explained variance was nearly equal to the models based on the SMM: 73% ($N = 100$; Figure 1c), 67% ($N = 1000$), and 21% ($N = 10{,}000$). In all cases the coefficient of variation approached a constant typically within a few thousand generations (Figure 2).

Because the rate of increase of md^2 with respect to divergence time was directly related to mutation rate, the coefficient of variation increased when multiple loci with differing mutation rates were used. However, incorporating the actual mutation rates into the calculation of md^2 using Equation 2 helped to offset some of this increased variation, decreasing the coefficient by as much as 20%. Similarly, when a length-dependent mutation rate was modeled, the coefficient of variation increased, but this was compensated for by the standardization coefficient, which incorporated the average size of the two alleles that an individual possessed (data not shown). In all cases, the standardized md^2 outperformed the unstandardized form.

Mean d^2, Heterozygosity, and Divergence Time

Typically md^2 was a better linear predictor of divergence time than heterozygosity over the entire range of divergence times. For example, when 10 loci with mutation rates of 10^{-3} were examined, md^2 remained linear throughout the range of divergence times (Figure 1a), whereas heterozygosity approached an asymptote after about 2000 generations (Figure 3). However, heterozygosity was a better predictor of divergence time over shorter time scales ($\leq$1000 generations) and over longer time scales when the mutation rate was low and the population size was large (Table 1).

Gene flow significantly reduced the precision of both md^2 and heterozygosity estimates of divergence time. For example, after 10,000 generations postdivergence with 1% gene flow, $N = 100$, and 10 loci with mutation rates of

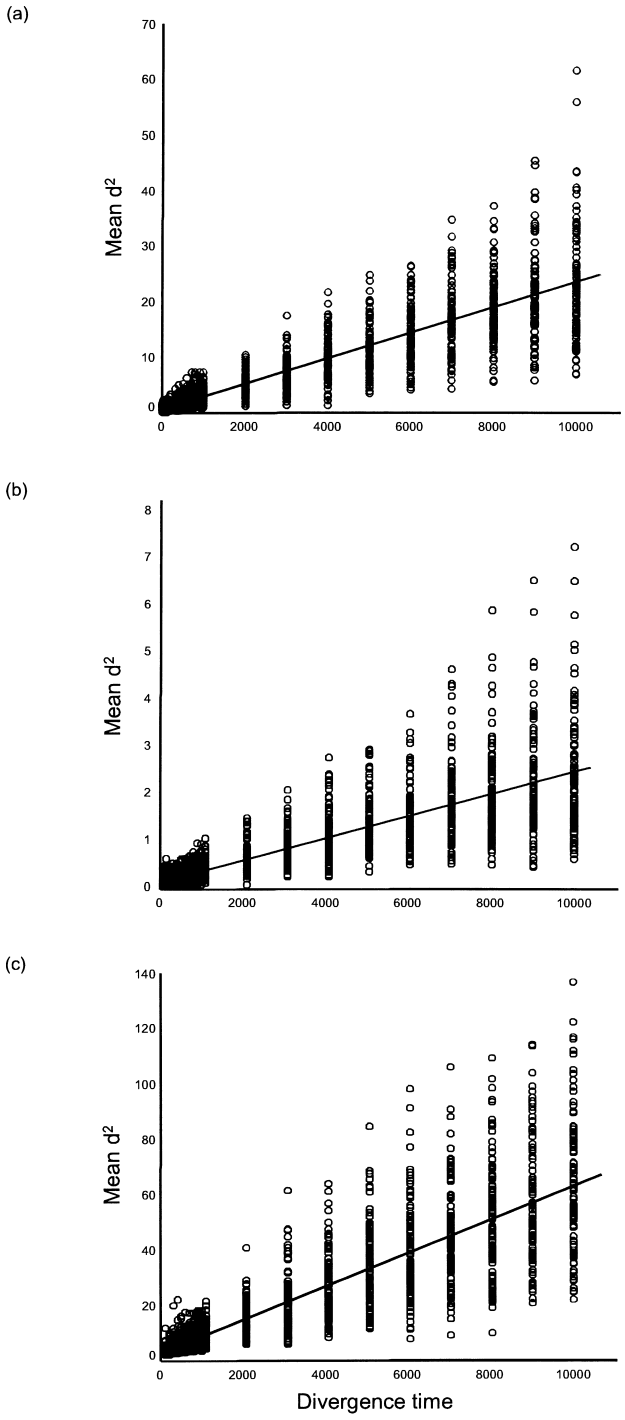

Figure 1. The relationship between md^2 of hybrid offspring and divergence time (t). Each data point represents an average based on 100 offspring generated from 1 of 100 pairs of populations each of 100 individuals. Ten loci were modeled with mutation rates of **(a)** 10^{-3} following the SMM ($md^2 = 0.42 + 0.0021t$), **(b)** 10^{-4} following the SMM ($md^2 = 0.041 + 0.00023t$), and **(c)** 10^{-3} following the TPM ($md^2 = 1.03 + 0.0061t$).

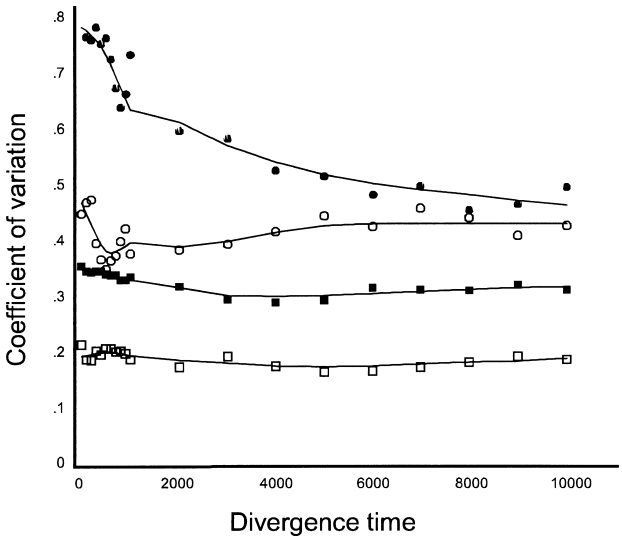

Figure 2. The relationship between the coefficient of variation in md^2 of hybrid offspring and divergence time. Each data point is based on 100 pairs of populations and 10 loci following the SMM with population size and mutation rates of 100 and 10^{-4} (filled circles), 100 and 10^{-3} (open circles), and 10,000 and 10^{-3} (filled squares), or 50 loci following the SMM and population size and mutation rates of 100 and 10^{-3} (open squares).

10^{-3}, the correlation coefficient between the average md^2 of within-population offspring and hybrid offspring only was 0.36, and for heterozygosity the coefficient was 0.56; for the same parameters but with $N = 1000$, the correlations were 0.05 and 0.35 for md^2 and heterozygosity, respectively.

Discussion

This article shows that md^2 is a linear measure of divergence time between parental lineages when there is no constraint on allele size. When there is a constraint on the maximum allele size, the duration of linearity between md^2 and divergence time can be predicted based on the mutation rate and mechanism (Goldstein et al. 1995). For example, when allele sizes are constrained to 50 repeats, under the stepwise mutation model, linearity is expected to be lost at approximately 200,000 and 2,000,000 generations for mutation rates of 10^{-3} and 10^{-4}, respectively. Under a two-phase mutation model with an average mutation size of two repeats instead of one, these times are halved. Because the duration of linearity increases to the square of the maximum allele size (Goldstein et al. 1995), microsatellite loci with slightly larger maximum allele sizes can be more informative over much longer times than those with smaller maximums.

Several transformations and modifications to the original formulation of md^2 have been proposed (e.g., Amos et al. 2001; Coltman et al. 1998; Hedrick et al. 2001; Pemberton et al. 1999). The results from this article show that variance in

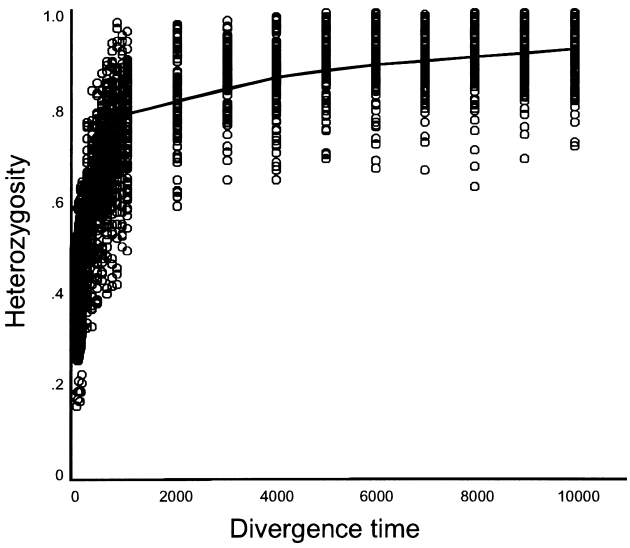

Figure 3. The relationship between multilocus heterozygosity of hybrid offspring and divergence time. Each data point represents an average of 100 offspring generated from 1 of 100 pairs of populations each of 100 individuals. Ten loci were modeled following the SMM with a mutation rate of 10^{-3}. The line represents a locally weighted regression.

md^2 increases with divergence time. For all parameter sets examined, the coefficient of variation approached a constant value, indicating that the variance increases proportionately to the average value of md^2. Thus a logarithm (+1) transformation is appropriate to restore homoscedasticity (Zar 1999). The transformed values are then related to divergence time by the logarithm function.

Standardization coefficients for md^2 that incorporate locus-specific mutation rates also are appropriate. The slope of the linear relationship between md^2 and divergence is directly proportional to mutation rate (Figure 1; see also Goldstein et al. 1995; Slatkin 1995). Thus d^2 values from different loci can be standardized using Equation 2. When the loci differ in mutation rates by an order of magnitude, the standardization can reduce the coefficient of variation by as much as 20%. Furthermore, if some loci mutate according to the SMM, while others mutate according to the TPM, then the average mutation size (in repeats, σ_l^2) can be incorporated into the standardization coefficient according to $\alpha_l = \mu_l \sigma_l^2$ (see Equation 2). When mutation rates are length dependent (Neff and Gross 2001; Webster et al. 2002; but see Xu et al. 2000), using the average of the two allele sizes possessed by each individual similarly reduces the coefficient of variation. Alternatively, the mean allele size at the locus could be used.

The mutation rate at a microsatellite locus can be inferred from the variance in allele sizes among individuals, and this measure of variance has been suggested as a standardization constant for md^2 (Hedrick et al. 2001). When sufficient data are available to precisely calculate the true variance in allele sizes, using variance as the standardization coefficient will be effective because it captures both mutation rate and mean mutation size (Di Rienzo et al. 1994; Neff et al. 1999). However, when limited genetic data are available to estimate the variance, or when the genetic data are from the same individuals used to calculate the md^2 values, some caution should be exercised. In these cases, important variation related to divergence, and not differential mutation rates, may be removed from the data (Hedrick et al. 2001). Provided the same loci are used across individuals, md^2 will accurately reflect the relative divergence times regardless of whether or not mutation rates vary and whether or not a standardization coefficient is utilized.

Table 1. Summary of correlations between md^2 or heterozygosity (H) and divergence time

	$N = 10^2$		$N = 10^3$		$N = 10^4$	
Comparison[a]	$\mu = 10^{-3}$	$\mu = 10^{-4}$	$\mu = 10^{-3}$	$\mu = 10^{-4}$	$\mu = 10^{-3}$	$\mu = 10^{-4}$
No migration						
W at 10^3:Hy at 10^3						
md^2	.90	.66	.50	.44	ns	ns
H	**.94**	**.73**	**.87**	**.60**	**.79**	**.48**
W at 10^4:Hy at 10^4						
md^2	**.98**	.92	.91	.90	.56	.53
H	.97	**.96**	**.96**	**.94**	**.94**	**.91**
Hy at 10^3:Hy at 10^4						
md^2	**.93**	**.89**	**.89**	.87	.55	.43
H	.78	.88	.87	**.89**	**.87**	**.85**

The numbers are Pearson correlation coefficients for three population sizes (10^2, 10^3, and 10^4) and two mutation rates (10^{-3} and 10^{-4}). Simulations are based on 100 populations of 10 loci following the SMM. Bold numbers denote the higher correlation coefficient of the pair and ns denotes a nonsignificant coefficient.

[a] Comparisons are between offspring from the same subpopulation (W = within population) and offspring from different populations (Hy = hybrid) at 10^3 or 10^4 generations postdivergence, or between hybrids at 10^3 generations postdivergence and hybrids at 10^4 generations postdivergence.

Overall md^2 is an excellent predictor of divergence time. As few as 10 loci can explain more than 73% of the variation for small populations. However, over time scales of up to 1000 generations postdivergence, heterozygosity outperformed md^2. Similarly, when populations were large (10,000 individuals), heterozygosity outperformed md^2 for time scales up to 10,000 generations; although heterozygosity of hybrid offspring did approach an asymptote after approximately 10^5 generations, at which point md^2 was more effective (data not shown). Thus heterozygosity appears better suited for short time scales, whereas md^2 appears better suited for longer time scales. As such, md^2 should be a better predictor of fitness than heterozygosity when fitness is affected by admixture of divergent lineages. This might be the case when there is population structuring and local adaptation where admixture can disrupt coadapted gene complexes (e.g., Hendry et al. 2000; Pemberton et al. 1999).

In conclusion, md^2 is a good predictor of divergence times of parental lineages over large time scales provided there is no gene flow between the lineages. Mean d^2 may be especially well suited to detecting outbreeding depression, whereas heterozygosity is better suited for detecting inbreeding depression. Finally, it is possible that heterogeneity in reported correlations between md^2 and fitness is partly due to historic differences in the degree of isolation of locally adapted lineages and detection of outbreeding versus inbreeding depression.

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